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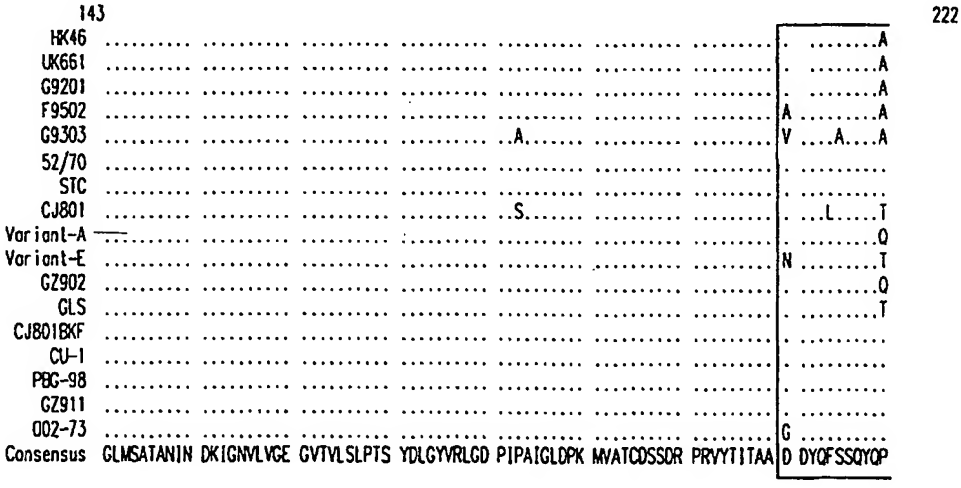

(57) Abstract

The invention relates to the generation and mutagenesis of recombinant infectious bursal disease virus by reverse genetics technology. Site-directed mutagenesis of certain amino acid residues on the Non-CEF adapted, very virulent strains of IBDV transforms the viruses into attenuated, CEF-adapted strains. The attenuated, CEF-adapted strains can be used as live vaccines against the very virulent strains. Curently available live attenuated vaccines were generated by serial passage through cell culture and the like, whereas the present invention provides a novel molecular mechanism in manipulation of the viral genome and the generation of attenuated vaccines by site-directed mutagenesis. The same approach can be used to produce vaccine strains from newly evolved IBDV viruses or to manipulate the antigenicity and pathogenicity of the virus.

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GENERATION OF RECOMBINANT INFECTIOUS BURSAL DISEASE VIRUSES BY REVERSE GENETICS TECHNOLOGY AND THE USE OF THE RECOMBINANT VIRUSES AS ATTENUATED VACCINES

RELATED APPLICATION

This application claims the priority of provisional patent application no. 60/098,684, filed on September 1, 1998.

FIELD OF THE INVENTION

This invention is in the field of molecular biology. This invention relates to the generation of recombinant infectious bursal disease virus and the manipulation of the viral genome by the reverse genetics technology. In particular, it relates to two DNA molecules comprising artificially constructed polynucleotide sequences substantially corresponding to all or a portion of infectious bursal disease d.s. RNA genome and the mutagenesis of certain amino acid residues of the virus so as to generate mutated attenuated vaccine strains against the virus.

BACKGROUND OF INVENTION

Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), is a highly contagious disease of young chickens, which results in significant losses to the poultry industry. After infection, IBDV multiplies rapidly in the developing B lymphocytes of the bursa of Fabricius, leading to immunosuppression and increased susceptibility to other diseases. Two distinct serotypes of IBDV, designated as serotype 1 and serotype 2, have been identified. The serotype 1 strains are pathogenic to chicken and vary in their virulence, whereas serotype 2 strains isolated from turkey, are apathogenic for both turkey and chicken. According to antigenic variation and virulence, serotype 1 strains can be divided into several groups: classical virulent strains, attenuated strains, antigenic variant strains and very virulent (vv) strains. Classical virulent strains cause bursal inflammation and severe lymphoid necrosis in infected chicken, resulting in immunodeficiency and moderate mortality. Generally, mortality peaks by the third day post-infection but death may still occur over the next 5 to 7 days resulting in a mortality up to 20%~30% in specific pathogen free (SPF) chickens. Attenuated strains have been adapted to chick embryo fibroblast (CEF) cells or other cell lines. They do not cause diseases in chickens, and therefore some of them are being used as live vaccines.

Antigenic variant strains were recognized by their ability to escape cross-neutralization by the antisera against classical strains. Chickens affected by the variant strains are characterized by severe atrophy of the bursa, without showing the inflammation symptom associated with the infection of classical strains (Vakharia et al. Virus Res 31:265-273, (1994)). Since the late eighties, the outbreaks of newly evolved, very virulent (vv) strains in Europe, Japan and China cause significant economical losses to the poultry industry. The vv strains can cause up to 60-100% mortality in SPF birds. These vv strains cause typical lesions of IBDV and are antigenically similar to the classical strains (Cao, Y. et al, Avian Dis. 42, 340-351, (1998)).

IBDV is a member of the Birnaviridae family, as its genome consists of two segments of double-stranded RNA (dsRNA). The smaller RNA segment (segment B) with a length of about 2.8kb, encodes VP1, a 90 kDa multifunctional protein with polymerase and capping enzyme activities. The larger RNA segment (segment A), with a length of about 3.2kb, encodes a polyprotein that is cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4. VP2 and VP3 are the major structural proteins of the virion, of which VP2 is the major host-protective immunogen of IBDV that contains the antigenic regions responsible for the induction of neutralizing antibodies (Brown, M. D. and Skinner, M. A. Virus Res, 40:1-15, (1996)). A second open reading frame (ORF), preceding and partially overlapping the polyprotein gene, encodes VP5, a 17kD polypeptide present in IBDV-infected cells. However, the function of the polypeptide is still unknown. The nucleotide sequences of genome segments A and B of various IBDV strains have been published, which conclude that the sequences of the major host-protective immunogen VP2 are highly conserved, except the central AccI-SpeI restriction fragment which has been designated as the hypervariable region.

Seven infectious bursal disease virus strains isolated from China have recently been characterized (Cao, Y. et al, Avian Dis. 42, 340-351, (1998)), including a classical strain CJ801, an attenuated strain GZ911, a variant strain GZ902, and four very virulent strains G9201, G9302, F9502 and HK46. Using reverse transcription-polymerase chain reaction (RT-PCR), the full length VP2 genes were amplified and the hypervariable regions were sequenced. Protein sequences of the hypervariable region (a.a. 143-382) of the field isolates confirmed their identities (Figure 1). CJ801 has the highest identity to the classical strains STC and 52/70. GZ902 has the highest identity to the American variant strains A, E and GLS

and they share unique amino acid residue at position 249K and 254S, which are not present in the other serotype 1 strains. Attenuated strain GZ911, like other cell culture-adapted strains, has substitutions at positions 279(D to N) and 284(A to T) as well as in the serine-rich heptapeptide region. Hence, these substitutions may play important roles in the reduced virulence of these strains. The four very virulent strains have the highest identity to the European very virulent strain UK661 and Japanese strain OKYM. They share unique amino acid residues at positions 222A, 256I, 294I, and 299S which are not present in other less virulent strains. The very virulent strains isolated in Guangdong (G9201, G9303) and Fujian (F9502) provinces have 1 to 5 amino acid substitutions at the two hydrophilic domains of VP2 comparing to UK661 and OKYM, indicating that new very virulent strains are evolving. Phylogenetic analysis suggests that Chinese very virulent IBDVs and European very virulent strains are derived from similar origin (Figure 2).

The principal methods of controlling IBD in young chickens are by vaccination with live attenuated strain of IBDV at the age of 0-5 weeks, or by in ovo vaccination, or by transferring high levels of maternal antibody induced by the administration of live and killed IBD vaccines to the breeder hens (Wyeth and Cullen, 1979). Both live attenuated virus vaccine and inactivated virus vaccine for this disease are available on the markets. The live attenuated vaccine available on the markets are generally derived by serial passage of classical or variant strains in embryonated SPF eggs, or in cells derived from embryonated SPF eggs , such as primary or secondary chicken embryo fibroblasts (CEF). Only the strains with their virulence reduced or eliminated by these methods can be used as live attenuated vaccines. Inactivated vaccine is prepared by culturing the virus itself, or by propagating the virus in SPF chicken, which are subsequently inactivated by heat, chemical treatment and the like. The attenuated strains derived from the classical strains and the variant strains can usually protect the birds from the attacks of the classical strains and the variant strains, respectively. However, these vaccine strains do not always offer 100% protection against the very virulent strains discovered in the recent years. Hence, it is important to develop an attenuated vaccine strains from the very virulent strains.

SUMMARY OF INVENTION

In general, attenuated live virus vaccines are preferred because they provoke a more long-lasting immune response (often both humoral and cellular), a good

mucosal immunity and are easy to produce. Presently live infectious bursal disease vaccines are developed by multiple passages of the field virus in chicken embryonic fibroblasts or in embryonated eggs. Hence, the method of producing live attenuated virus is a random, uncontrolled process and as a result, these vaccines suffer from a number of drawbacks. Firstly, a field virus may not be adapted to CEF after many attempts and hence no live attenuated virus could be derived. Secondly, even some of the field viruses can be adapted in CEF, a population of viruses of different characteristics and different degree of virulence may be generated. Because uncontrolled mutations are introduced into the viral genome during serial passages, a population of virus particles heterogeneous in their virulence and immunizing properties are generated. Single virus has to be cloned and the pathogenicity of each cloned virus has to be tested. Thirdly, it is possible that such traditional attenuated live virus vaccines can revert to virulence, resulting in disease of the inoculated animals and the possible spread of the pathogen to other animals.

In this invention, the development of techniques for controlled manipulation of genetic material has allowed the creation of CEF-adapted virus vaccines which avoids the disadvantages of the attenuated virus vaccines generated by conventional techniques.

The present invention involves the generation of full length cDNA from the double strand RNA genome of the a very virulent IBDV strain by reverse transcription. The two cDNAs were individually cloned into eukaryotic expression vectors before carrying out site-directed mutagenesis. The transfection of the mutated expression vectors into chicken embryo fibroblasts allowed the regeneration of a CEF adapted, live virus, whereas the transfection of the non-mutated expression vectors could not generate any live virus.

The present invention provide a novel technique that creates CEF-adapted IBDV strains by site-directed mutagenesis which can be used for the preparation of live and inactivated vaccines against IBDV infection. The mutant viruses being attenuated in a controlled manner can elicits a strong immune response in host animals.

It is another object of the present invention to create new live vaccine strains from classical, variant, very virulent or any other newly evolved IBDV strains.

It is another object of the present invention to provide a mutant IBDV which can be used not only for the preparation of a vaccine against IBDV infection but also against other infectious diseases.

It is a further object of the present invention to produce subunit vaccines, pharmaceutical and diagnostic preparations comprising a heterologous polypeptide expressed by an IBDV mutant according to the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and elements of the present invention will be better understood from the following detailed description of preferred embodiments of the invention in which:

Fig. 1 shows an alignment of the hypervariable regions of various ISDV strains.

Fig. 2 shows the phylogenetic tree showing the relationship between various IBDV strains.

Fig. 3 shows construction of plasmid FA-pBssK containing full-length cDNA copy of segment

Fig. 4 shows construction of plasmid FB-pBssK containing full-length cDNA copy od segment B of IBDV HK46 strain.

Fig. 5 shows changes in Bursa Weight after virus inoculation.

DETAILED DESCRIPTION

From a previous publication of our group (Cao, Y. et al, Avian Dis. 42, 340-351, (1998)), the hypervariable region of the VP2 protein of seven Chinese IBDV strains were characterized. By comparing the sequences (Fig.1), several amino acid residues on the VP2 protein were postulated to be responsible for the attenuation of the virus or the antigenicity of the virus. Based on these data, this invention developed a molecular procedure that can be used to engineering IBDV genome in a controlled manner, which can change the property of the virus, such as generating a live attenuated strain of specific antigenicity.

The invention comprises of the RNA genomes derived from a very virulent IBDV strain HK46, including a biological pure RNA encoding SEQ ID NO:1, which encodes a 1012 amino acids long polypeptide, and a biological pure RNA encoding SEQ ID NO:2, which encode a polypeptide of 879 amino acids long. The RNA genomes were reverse transcripted into cDNAs which were then subcloned into various vectors, of which some of them containing an eukaryotic promoter, such as CMV promoter or RSV promoters. The transfection of two of these vectors, which

individually carries the segment A cDNA and segment B cDNA of the very virulent strain HK46, into CEF together, can not yield a viable virus.

A preferred IBDV mutant according to the invention can propagate in CEF or cell lines as a result of point mutation and/or a deletion and/or insertion of an oligo- or polynucleotide sequence in the gene encoding VP2.

Preferably, the mutation can be introduced in the IBDV segment A genomic region encoding the VP2 having an amino acid sequence (SEQ ID NO:32) shown in SEQ ID NO:1. The nucleic acid sequence of the whole segment A genome was determined and is shown in SEQ ID NO:1.

In addition, the mutation can be introduced in the IBDV segment B genomic region encoding the VP1 having an amino acid sequence (SEQ ID NO:33) shown in SEQ ID NO:2. The nucleic acid sequence of the whole segment B genome was determined and is shown in SEQ ID NO:2.

Certain amino acid residues encoded by the segment A cDNA inserts cloned into the vectors can be mutated by site-directed mutagenesis or PCR at one or more amino acid residues at positions 222, 249, 254, 256, 279, 284, 294, 299, 326 to 332 and combinations thereof. The transfection of certain mutated vectors together with the vector carrying the segment B cDNA insert into CEF can yield viable viruses, which are in the embodiment of this invention.

In a particular preferred embodiment of the invention, the viable mutant virus consists of substitutions at the amino acids residues 249(Q--> K) and 254(G-->S) of segment A protein, which can be passaged in CEF.

In another particular preferred embodiment of the invention, the viable mutant virus consists of substitutions at the amino acids residues 279(D-->N), 254(A-->T) of segment A protein, which can be passaged in CEF.

According to the present invention such mutant IBDVs are generated in a controlled, non-random manner. The mutation introduced into the viruses are site-directed and the mutations can result in the change of the virulence and antigenicity of the virus.

Although, the IBDV mutant according to the invention is derived from strain HK46, an isolate recovered from infected tissues of 6 week old broilers by the Agricultural and Fishery Department of the Hong Kong Special Administration Region, any IBDV strain can be used to prepare the IBDV mutant, e.g. the very virulent strains G9201, G9303, F9502, the classical CJ801 and the variant strain

GZ902 described in a publication (Cao, Y. et al, Avian Dis. 42, 340-351, (1998)), or any newly evolved virulent strains.

From the cDNA derived from the genome segment A of any other IBDV, the NdeI-SpeI restriction fragment (nucleotides 652-1192) can be subcloned into the vectors containing SEQ ID NO:1. The vectors generated thereof can be used for mutagenesis as described above to generate cell culture-adapted, attenuated virus.

IBDV mutants according to the invention can also be obtained by inserting a nucleic acid sequence into the VP2 coding region. Such a nucleic acid sequence can inter alia be an oligonucleotide, for example of about 10-90 bp, or a gene encoding a polypeptide. Said nucleic acid sequence can be derived from any source, e.g. synthetic, viral, prokaryotic or eukaryotic.

The live recombinant vaccine generated according to this invention is administered in amounts sufficient to stimulate the immune system to confer resistance to IBD. The vaccine according to the invention is administered in such amount that will induce immunity in a chicken against challenge by a virulent IBDV. Immunity is defined as the induction of a significant level of protection in a population of chickens after vaccination compared to an unvaccinated group. A dose of 10^2 to 10^5 EID_{50} of the IBDV mutant per chicken is recommended in general. The vaccine according to the invention may also be in a lyophilized form.

In addition to the IBDV recombinant vaccine described above, a vaccine according to the invention also comprises a pharmaceutically acceptable carrier compatible with the IBDV recombinant vaccine. As a pharmaceutically acceptable carrier a sterilized isotonic solution such as a physiological saline and a phosphate buffer may be added to the IBDV recombinant vaccine. Further suitable excipients are skimmed milk, glycerol, dextrose, sorbitol, starch, mannitol and the like. In addition, if desired, the vaccine can contain amounts of auxiliary substances which enhance the effectiveness of the vaccine, such as emulsifiers and adjuvants. Adjuvants such as aluminum hydroxide, aluminum phosphate, plant and mineral oil and the like, are administrated with the vaccine in amounts adequate to enhance the immune response to the IBDV. The amount of adjuvant added to the vaccine will vary dependent on the nature of the adjuvant.

For administration to animals, the live IBDV recombinant vaccine can be given to the animal orally, nasally, ophthalmically, intradermally, subcutaneously, intramuscularly or by aerosol, spray and drinking water. It can also be given to fertilized eggs by in ovo injection. It is preferred to vaccinate the chickens by mass

administration techniques such as by placing the vaccine in drinking water or by spraying the vaccine.

An live IBDV recombinant vaccine according to the invention can also be used to prepare an inactivated vaccine, which can be administered by injection. Parenteral administration of the inactivated vaccine can be carried out intradermally, subcutaneously, intramuscularly.

The recombinant vaccine generated by this invention is administered to poultry, including breeders, broilers, chickens, hens, layers, roasters, roosters and turkeys. Preferably, the vaccine is administrated to the poultry to prevent IBD before birth, and after hatching.

EXAMPLES

Example 1

Propagation and Purification of IBDV from Bursae

The very virulent strains of infectious bursal disease virus (IBDV), HK46, G9303, G9201, and F9502, which were isolated in the southern part of P R China, were propagated in SPF chickens at 5 weeks of age. At 72 hr post-infection, the bursa were removed and homogenized in TNE buffer (10 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH8.0). After freezing and thawing for three times, the homogenates were centrifuged at 14 000 rpm for 15 min at 4°C, and the supernatants were collected for virus purification. One ml of bursal homogenate supernatants were loaded onto 4 ml of 40% sucrose cushion and ultracentrifuged at 55,000 g (Beckman sw55Ti rotor, 22,000 rpm) for 2.5 hr at 4°C. After removing the cushion, the virus particle pellets at the bottom were stored at -20°C.

Example 2

Extraction of Viral RNA

Purified IBDV particles were resuspended in proteinase buffer (TNE + 0.5%SDS), digested with 1 mg/ml proteinase K for 1 hr at 37°C, and extracted with phenol-chloroform-isoamylalcohol (25:24:1, V/V) twice. Finally the viral dsRNA were precipitated from the upper aqueous-phase by ethanol.

Example 3

Synthesis of cDNA by Reverse Transcriptase

The cDNA of IBDV segments A and B of the very virulent strain, HK46, were synthesized independently. Two primer pairs, designated as A5 (SEQ ID NO: 3) with EcoR I site and A3 (SEQ ID NO: 4) with Kpn I site, B5 (SEQ ID NO: 5) with EcoR I site and B3 (SEQ ID NO: 6) with Xba I site, were used for synthesizing cDNA of IBDV segments A and B. Viral ds RNA mixed with primers A5 and A3 (for segment A) or B5 and B3 (for segment B) were denatured by boiling for 5 min and cooled on ice for 2 min. After adding equal volume of 2X PCR buffer containing 10 mmole of dNTPs, 50 mmole of $MgCl_2$, 200 mmole of DTT and 200 U of SuperScript II RT (life Technologies), a total volume of 50 ul of reaction mixture was incubated at 50°C for 1 hr to synthesize the first strand cDNA. The reaction was terminated at 70°C for 15 min and the ds RNA was then digested by 1 U of RNase H at 37°C for 20 min.

Example 4

PCR Amplification of the Whole IBDV Genome

All primers used for PCR and mutagenesis are listed in Table 1. Four fragments, designated as FA5 (5' end fragment of segment A), FA3 (3' end fragment of segment A), FB5 (5' end fragment of segment B), FB3 (3' end fragment of segment B), were independently amplified by Expand™ High Fidelity PCR System (Boehringer Mannheim), using the first strand cDNA as templates. The amplifications were performed using Robocyclerâ Gradient 96 (Stratagene) following the program of 94°C for 3 min; 30 cycles of 94°C for 40 sec, 60°C (for FA5 and FA3 amplification) or 52°C (for FB5 and FB3 amplification) for 40 sec, 72°C for 2 min 30 sec; and finally 72°C for 10 min. Four primer pairs were used for amplifications of the corresponding fragments, in which A5 (SEQ ID NO:3) and A3AP (SEQ ID NO:7) were used for amplifications of fragment FA5, A5SP (SEQ ID NO:8) and A3 (SEQ ID NO:4) for fragment FA3, B5 (SEQ ID NO:5) and B3AP (SEQ ID NO:9) for fragment FB5, B5SP (SEQ ID NO:10) and B3 (SEQ ID NO:6) for fragment FB3.

TABLE 1. Primers for genome cloning and mutagenesis of IBDV

SEQ IDNO:	Name	Size (bp)	RE site	direction	location	sequence
3	A5	40	Eco R I	+	A,5' end	5'ATGAATTCAGGATACGATCGGTCTGAC CCCGGGGGAGTCA3'
4	A3	33	Kpn I	-	A,3' end	5'TAGGTACCAAGGGACCCGCGAACGGAT CCAATT3'
5	B5	29	Eco R I	+	B,5' end	5'TTAGAATTCTAGGATACGATGGGTCTGA C3'
6	B3	29	Xba I	-	B,3' end	5'ATTTCTAGATGGGGGCCCCCGCAGGCG AA3'
7	A3AP	27	Sal I	-	A, 1729-1755	5'CAGGTGAAGCARAGAATCCCGTCGACT A3'
8	A5SP	28	Sal I	+	A, 1718-1745	5'CCAGAATCCYGTAGTCGACGGGATTCTT 3'
9	B3AP	21	Bgl II	-	B, 1848-1868	5'GATCCCRAGATCTTTGCTGTA3'
10	B5SP	24	---	+	B, 1757-1780	5'CCTTGCACAACCAGGGTACCTGAG3'
11	NTA	34	---	-	A, 956-989	5'GGTCGTTAGCCCATTGTTTCGGGCCACA GCTCTG3'
12	NTS	38	---	+	A, 969-1006	5'GCAAACAATGGGCGAACGACCGGCACT GACAACCTTAT3'
13	KSA	35	---	-	A, 866-900	5'GGCTTTGGACGCTTGTTTTAAACACGAG CTCTCCC3'
14	KSS	33	---	+	A, 881-913	5'TAAAACAAGCGTCCAAAGCCTTATACTT GGGTGC3'
15	33OR	27	---	-	A, 1115-1141	5'pACTGCTAGGCTCCCTCTTGCTGACCAT 3'
16	mut-KS	35	---	-	A, 873-907	5'pAGTATAAGGCTTTGGACGCTTGTTTTA AACACGAG3'
17	mut-NT	36	---	-	A, 963-998	5'pGTCAGTGCCGGTCGTTAGCCCATTGTT TGCGGCCAC3'

R = A or G; Y= C or T

Example 5

Construction of Full Length cDNA Clones of IBDV Genome

Full-length cDNA clones of IBDV segments A and B genome were independently prepared. To obtain cDNA clones of segment A of HK46 strain, two cDNA fragments FA5 and FA3 were amplified by PCR using two primer pairs, A5 (SEQ ID NO:3) and A3AP(SEQ ID NO:7), A5SP(SEQ ID NO:8) and A3(SEQ ID NO:4) and using first strand cDNA of segment A as template. Fragment FA5 digested with EcoR I and Sal I was cloned into the EcoR I/Sal I site of pBssK vector (Strategene) to obtain plasmid FA5-pBssK. After digesting fragment FA3 and plasmid FA5-pBssK with Sal I and Kpn I, the insert (FA3) and the vector (FA5-pBssk) were ligated with T4 DNA ligase. After transforming the ligated DNA into E.coli XL1-Blue strain, positive colonies were screened by PCR. Plasmid DNAs are extracted from positive colonies by a mini-prep kit (Wizard Plus Minipreps DNA purification system, Promega) and their identities were confirmed by digestion with EcoR I and Kpn I. The size of the plasmid FA-pBssK is about 6.2kb. This plasmid contains a full-length cDNA copy of segment A of IBDV HK46 (Fig.3).

To obtain cDNA clones of segment B of HK46, two fragments FB5 and FB3 were amplified by PCR using two primer pairs, B5 (SEQ ID NO:5) and B3AP(SEQ ID NO:9), B5SP(SEQ ID NO:10) and B3(SEQ ID NO:6), respectively, and using first strand cDNA of segment B as template. There is an unique Bgl II site in the overlapping region of fragments FB5 and FB3. Because pBssK vector lacks Bgl II site, another plasmid pBssK-Bgl, in which an unrelated cDNA containing Bgl II sites was cloned into the EcoR I site of pBssK, was employed to construct full-length cDNA clones of fragment B. Fragment FB3 digested with Bgl II and Xba I was cloned into the Bgl II/Xba I site of plasmid pBssK-Bgl to obtain plasmid FB3-pBssK. After digesting fragment FB5 and FB3- pBssK with EcoR I and Bgl II, the insert (FB5) and the vector (FB3-pBssk) were ligated with T4 DNA ligase. After transforming the ligated DNA into E.coli XL1-Blue strain, positive colonies were screened by PCR. Plasmid DNAs were extracted from positive colonies by a mini-prep kit (Wizard Plus Minipreps DNA purification system, Promega) and their identities were confirmed by digestion with EcoRI and XbaI. The size of the plasmid FA-pBssK is about 5.7 kb. Therefore this plasmid contains a full-length cDNA copy of segment B of IBDV HK46 strain, but does not contain the unrelated cDNA fragment (Fig.4).

Example 6

DNA Sequencing

PCR sequencing reactions were carried out using an Autocycle DNA Sequencing Kit (Pharmacia) following the manufacturer's recommended conditions. Specific primers with fluorescein at 5' end, as listed in table 2, were used for the sequencing. Sequence determination was performed using Automated Laser Fluorescein (ALF) nucleotide sequencer (Pharmacia). Sequences data were assembled and analyzed using the GCG package (Genetics Computer Group, Madison, Wisconsin, USA). Phylogenetic trees were prepared by the UPGMA method (Unweighted Pair Group with Arithmetic Mean)of the GeneWorks program (IntelliGenetics Inc, Mountain View, CA, USA).

TABLE 2. Primers for DNA sequencing

SEQ IDNO:	Name	Size (bp)	direction	location	Sequence
18	A639S	19	+	639-657	5'AGCTTACCCACATCATATG3'
19	A723A	17	-	723-739	5'TCACTGCTGTCACATGT3'
20	A768S	20	+	768-787	5'GCCGATGATTACCAATTCTC3'
21	A1041S	17	+	1041-1057	5'ACCCAGCCAATCACATC3'
22	A1405S	16	+	1405-1420	5'CGGAGTACTTCATGGA3'
23	A1835S	16	+	1835-1850	5'GGAAGACGCCATGACA3'
24	A2174S	18	+	2174-2191	5'CTATGGCGAGATTGAGAA3'
25	A2538S	17	+	2538-2554	5'ATGGCCAACTTCGCACT3'
26	B296S	17	+	296-312	5'CAAGATTCTGCAGCCAC3'
27	B429S	17	+	429-446	5'CAGGAGTACTTCCCAAA3'
28	B876S	19	+	876-894	5'AACCTCAAGTCATCAAGTG3'
29	B1029A	17	-	1029-1045	5'TCACTTAGCATGCTGAG3'
30	B1229S	19	+	1229-1247	5'GTCACTCTACAAGTTCAAC3'
31	B2568A	17	-	2568-2584	5'GTCTGTGGGTTCTTAAC3'

Example 7

Site-directed Mutagenesis by PCR

According to the results of sequence analysis of VP2 cDNA of IBDV, amino acid substitutions at position 279 (D®N) and 284 (A®T), as well as in the serine-rich heptapeptide region (330 S®R), may result in the reduced virulence of classical IBDV strains. In addition, two other specific amino acid residues, 249K and 254S, may be the signature residues of the variant IBDV strains. So three combinations of mutations were introduced to the segment A cDNA of strain HK46: (a) Mutant NT: substitutions at nucleotide positions 966 (G®A) and 981 (G®A) can result in amino acid substitutions at residues 279(D®N) and 284(A®T); (b) Mutant KS: nucleotide substitutions at positions 876 (C®A) and 891 (G®A) can result in amino acid substitutions at residues 249(Q®K) and 254 (G®S); (c) Mutant R: nucleotide substitution at position 1121 (T®A) can result in amino acid substitution at position 330 (S®R).

To introduce the mutation sequences into segment A of IBDV, plasmids NT-FA-pBssK and KS-FA-pBssK were constructed by oligo-nucleotide directed mutagenesis, or by using specific primer pairs and FA-pBssK as template in PCR reactions. To construct plasmid NT-FA-pBssK, two primer pairs, designated as A5 (SEQ ID NO: 3) and NTA (SEQ ID NO: 11), NTS (SEQ ID NO: 12) and A3AP (SEQ ID NO: 7), were used to amplify two DNA fragments of 991 and 786 bp, respectively. These two DNA fragments were purified, combined and reamplified by PCR using the primers A5 and A3AP. Pfu polymerase (Strategene) was used in the PCR reaction to increase the fidelity. To increase the yield of the combined fragment, five cycles of 94°C for 2 min, 50°C for 1 min and 72°C for 2 min were run without adding any primers in the PCR cocktails. Subsequently, the primer pair A5 and A3AP was added into the PCR mixtures, and the amplification was performed following the program of 94°C for 3 min, 30 cycles of 94°C for 40 sec, 60°C for 40 sec, 72°C for 2 min 30 sec, and finally 72°C for 10 min. After amplification, the PCR product of 1756 bp were then digested with EcoR I and Sal I. The resulting fragment of 1726 bp was cloned into the EcoR I / Sal I site of FA-pBssK to substitute the EcoR I/Sal I insert of the plasmid FA-pBssK to obtain the plasmid NT-FA-pBssK. The mutated sequences were confirmed by cycling PCR sequencing. The plasmid NT-FA-pBssK contains full-length cDNA of segment A of HK46 strain but has substitutions at nucleotide positions 966 (G®A) and 981 (G®A).

To construct plasmid KS-FA-pBssK, two primer pairs, A5 (SEQ ID NO: 3) and KSA (SEQ ID NO: 13), KSS (SEQ ID NO: 14) and A3AP(SEQ ID NO: 7), were used to amplify the DNA fragments of 902 and 874 bp respectively, using FA-pBssK as template. A PCR product of 1756 bp was then amplified from the two PCR products as described above, which was subsequently cloned into the EcoRI/SalI site of the plasmid FA-pBssK. The mutated sequences were confirmed by cycling PCR sequencing using sequencing primers. The new plasmid KS-FA-pBssK contains full-length cDNA of segment A of HK46 but has substitutions at nucleotide positions 876 (C®A) and 891 (C®A).

Example 8

Mutation of the Serine-rich Heptapeptide Region on VP2 Using Altered Sites II Mammalian Mutagenesis System

Carrying the genes for ampicillin / chloramphenicol resistance and CMV I.E. enhancer / promotor, the pALTER-MAX vector may be used for mutagenesis and expression of genes in eukaryotic cells (Promega). In this study, the segment A cDNA (SEQ ID NO:1) of strain HK46 was subcloned into the pALTER-MAX vector. The plasmid FA-pBssK was digested with EcoR I, Kpn I and Bgl I. Because there is an unique Bgl I site in the pBssK vector but not in the sequence of fragment FA, the fragment FA and pBssK can easily be separated by 1% agarose gel after the plasmids were digested with Bgl I. After digestion, the FA fragment (3269 bp) was purified and cloned into the EcoR I/Kpn I site of pALTER-MAX vector to create plasmid FA-pALTER.

To substitute serine at amino acid residue 330 with arginine, the nucleotide T at position 1127 was changed to A using Altered sites II mammalian mutagenesis system (Promega). The single strand (ss) DNA of plasmids FA-pALTER and was produced using the methods recommended by supplier. A primer designated as 330R (5'ACTGCTAGGCTCCCTCTTGCGGA CCA3') that was phosphorylated at the 5'-end was synthesized and used for producing a mutation on plasmid FA-pALTER in which the nucleotide 1127(T) was substituted with A. Twenty microliters of annealing reaction mixture containing 0.05 pmole of ssDNA of FA-pALTER, 1.25 pmole of primer 330R, 0.25 pmole of ampicillin repair oligonucleotide, 20mM Tris-HCl (pH7.5), 10 mM $MgCl_2$ and 50 mM NaCl was heated to 75°C for 5 min, and then cooled slowly down to room temperature. After adding 3 ul of synthesis buffer containing 100 mM Tris-HCl (pH7.5), 5 mM dNTPs, 10 mMATP and 20 mM DTT,

ten units of T_4 DNA polymerase and 3 units of T4 DNA ligase were mixed with annealing reaction mixture, and the total volume of DNA synthesis reaction was adjusted to 30 ul with deionized water. Subsequently, synthesis mixture was incubated at 37°C for 2 hr to perform mutant strand synthesis and ligation. After incubation, the synthesis mixture was transformed into E coli strain ES1301 mutS competent cells by electroporation. The transformed ES1301 mutS cells were resuspended in 1 ml of LB broth without any antibiotics, and incubated at 37°C for 3 hr. By adding 0.5 ml of transformed cell culture to 4.5 ml of selective medium (LB broth with 100 ug/ml ampicillin), the transformed ES1301 mutS cells were cultured at 37°C with shaking at 250rpm overnight. Plasmid DNA was then extracted by the Wizard® Plus Minipreps DNA purification system and then transformed into Ecoli strain JM109 competent cells. Ten colonies were screened by direct sequencing with primer A768S (SEQ ID NO: 20) using an automatic sequencer. The resulting mutant plasmid designated as R-FA-pALTER was obtained.

Example 9

Generation of Recombinant viruses by Transfection

The segment A cDNAs from plasmids FA-FA-pBssK, NT-FA- pBssK, KS-FA-pBssK and R-FA-pALTER were released by EcoR I, Kpn I and Bgl I digestion and then subcloned into a plasmid at a site downstream to a CMV promoter. To clone the fragment FB into this plasmid, the plasmid FB-pBssK was digested with three restriction enzymes EcoR I, Xba I and Pvu II, where Pvu II can only digest pBssK vector but can not digest fragment FB. Subsequently the fragment FB was purified and inserted into the EcoR I/ Xba I site of the plamid carrying a CMV promoter. This plasmid contained the full-length cDNA copy of segment B downstream to a CMV promoter.

The CEFs were grown to 80% confluence in a 12-well tissue culture plate (Falcon) and washed once with phosphate-buffered-saline (PBS). One ml of serum free EME medium (SF-EMEM) (Life Technologies) were added to monolayers of each well, and the cells were incubated at 37°C for 1 hr in 5% CO_2 incubator. 5ul (0.2ug/ul) of both plasmids, CMV-FA and CMV-FB were then transfected to the CEF by Lipofectamine (Life Technologies), according to the manufacturer's procedures. After 5 hr of incubation at 37°C, the transfection mixture was replaced with EMEM containing 10% FBS, and the cells were further incubated at 37°C in 5% CO_2 incubator for desired time intervals. The plasmids containing mutants of segment A

cDNAs (KS-FA or NT-FA or R-FA) and/or CMV-FB were also transfected into CEF using the same procedure described above. After 96 hours, cytopathic effect was observed. The ability of the recombinant viruses in propagating in CEF was further tested by adding 0.1 ml virus suspension collected 96 hr posttransfection into a well of a 12 well plate containing 80% confluent CEF with 1 ml cell culture medium. The results on transfection and virus propagation are summarized in Table 3.

TABLE 3. CPE on CEF momolayers transfeced by plasmids containing full-length cDNA of both segments A and B of HK46 strain or its mutants. The presence of virus was tested by passages in CEF cells.

	R1	R2	NT	KS	FA
Transfection I: (96 hrs after transfection)	+	+	+	+	-
Transfection II (96hrs after transfection)	+	+	+	+	-
Passage one in CEF (with transfection I)	-	-	+	+	-
Passage two in CEF	-	-	+	+	-

R1, R2: Transfected with two independent CMV-R plasmids and CMV-FB plasmid.
NT: Transfected with CMV-NT-FA plasmid and CMV-FB plasmid.
KS: Transfeced with CMV-KS-FA plasmid and CMV-FB plasmid.
FA: Transfected with wild-type CMV-FA plasmid and CMV-FB plasmid.

Example 10

Confirmation of antigenic sequence of the recombinant virus by DNA sequencing

After virus propagation in CEF, cell debris was removed from the supernatant by centrifugation at 14,000rpm for 20min at 4°C. One ml of supernatant was loaded onto 4 ml of 40%(v/v) sucrose cushion and ultracentrifuged at 55,000g (Beckman sw55Ti rotor, 22,000rpm) for 2.5 hr at 4°C. The virus at the pellet was processed as stated in Examples 3&4 to produce first stranded cDNA. PCR reaction as stated in Example 5 was carried out to amplify the 5'cDNA segment of genome A (equivalent to FA5), which was then sequenced to confirm the mutation (Table 2).

Example 11

Animal tests

The recombinant virus (HK46-NT) generated from transfection was passaged and titred in CEF. To compare its pathogenicity with its parent strains, 3.2x105TCID50 HK46-NT and 1.6x105EID50 HK46 were administrated to 4 weeks old SPF birds. No birds vaccinated with HK46-NT showed any symptomes nor mortality, whereas all birds challenged by the parent strain HK46 were sick and the mortality reached 60%. The weights of the bursa after chanllenge were recorded in figure 5. To test the immunologicity of HK46-NT, it was administrated to SPF chickens at 25 days old (106TCID50/bird) and antibody titre at 43 days old was measured by a commercial ELISA kit (Idexx Inc.). The signal-to-noise (S/N) ratios were 3.41+/- 0.68 and 1.37+/-0.13, respectively.

While the present invention has been described in a number of different exemplary embodiments, it will be understood that the principles of the invention can be extended to still further embodiments and that the embodiments illustrated here are not intended to limit the scope of the invention as set forth in the appended claims.

WHAT IS CLAIMED IS:

1. A biological pure RNA encoding SEQ ID NO:1 or a fragment thereof of from 1 to 1012 amino acids long; or a biological DNA encoding to said biological pure RNA. (v.v. IBD segment A)

2. A biological pure RNA encoding SEQ ID NO:2 or a fragment thereof of from 1 to 879 amino acids long; or a biological DNA encoding to said biological pure RNA. (v.v. IBD segment B)

3. The biological pure DNA of claim 1, consisting of SEQ ID NO:1.

4. The biological pure DNA of claim 2, consisting of SEQ ID NO:2.

5. The biological pure DNA of claim 3, consisting of one or more nucleotide substitutions generated by mutagenesis using molecular approaches.

6. The biological pure DNA of claim 4, consisting of one or more nucleotide substitutions generated by mutagenesis using molecular approaches.

7. The biological pure DNA of claim 3, consisting of one or more nucleotide substitutions resulting in point mutations at one or more amino acid residues at positions 222, 249, 254, 256, 279, 284, 294, 299 and 326 to 332.

8. A recombinant vector consisting of the biological pure DNA of claim 5.

9. A recombinant vector consisting of the biological pure DNA of claim 6.

10. A recombinant vector consisting of the biological pure DNA of claim 7.

11. The generation of recombinant infectious bursal disease virus by the co-transfection of the recombinant vectors of claims 8 and 9.

12. The generation of recombinant infectious bursal disease virus by the co-transfection of the recombinant vectors of claims 9 and 10.

13. An recombinant infectious bursal disease virus generated by the procedures described in claim 11, comprising mutations at one or more of the amino acid residues, wherein the IBDV mutant acquire the ability of propagation in chicken embryo fibroblasts, or other primary tissue culture or cell lines.

14. An recombinant infectious bursal disease virus generated by the procedures described in claim 12, comprising mutations at one or more of the amino acid residues, wherein the IBDV mutant acquire the ability of propagation in chicken embryo fibroblasts, or other primary tissue culture or cell lines.

15. An recombinant infectious bursal disease virus generated by the procedures described in claim 11, comprising mutations at one or more of the amino acid residues at positions 249, 254, 279, 284, 326 to 332 of the SEQ ID NO:1, wherein the IBDV mutant acquires the ability of propagation in chicken embryo fibroblasts, or other primary tissue culture or cell lines.

16. An recombinant infectious bursal disease virus generated by the procedures described in claim 12, comprising mutations at one or more of the amino acid residues at positions 249, 254, 279, 284, 326 to 332 of the SEQ ID NO:1, wherein the IBDV mutant acquires the ability of propagation in chicken embryo fibroblasts, or other primary tissue culture or cell lines.

17. An IBDV mutant according to claim 13, wherein the mutation is selected from the group consisting of a deletion, an insertion, and a deletion and insertion and point mutations.

18. An IBDV mutant according to claim 15, wherein the mutation is selected from the group consisting of a deletion, an insertion, and a deletion and insertion and point mutations.

19. An IBDV mutant according to claim 13, wherein at least one heterologous nucleic acid fragment from a virus or a pathogen encoding a polypeptide is inserted into the VP2 locus.

20. An IBDV mutant according to claim 15, wherein at least one heterologous nucleic acid fragment from a virus or a pathogen encoding a polypeptide is inserted into the VP2 locus.

21. A cell infected with an recombinant IBDV mutant according to claim 13.

22. A cell infected with an recombinant IBDV mutant according to claim 15.

23. A cell infected with an recombinant IBDV mutant according to claim 17.

24. A cell infected with an recombinant IBDV mutant according to claim 19.

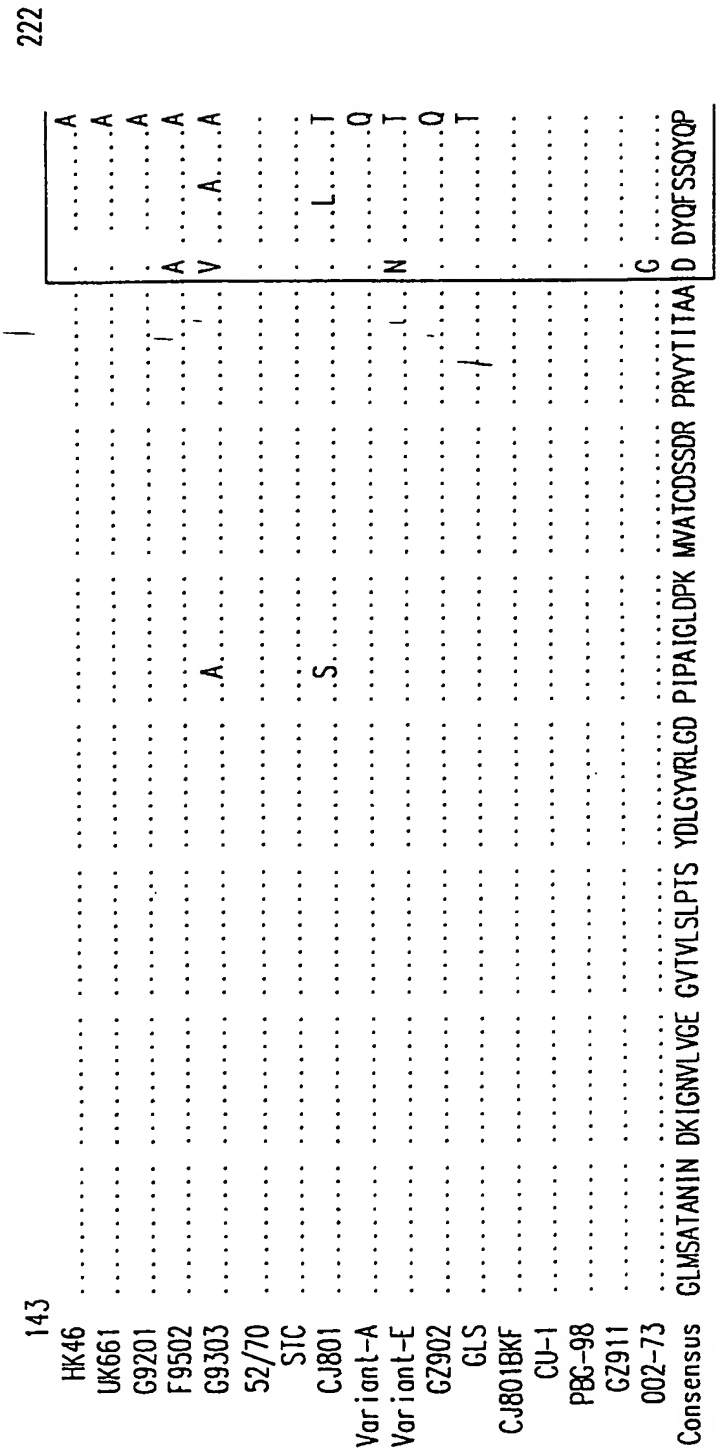
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FIG.1A

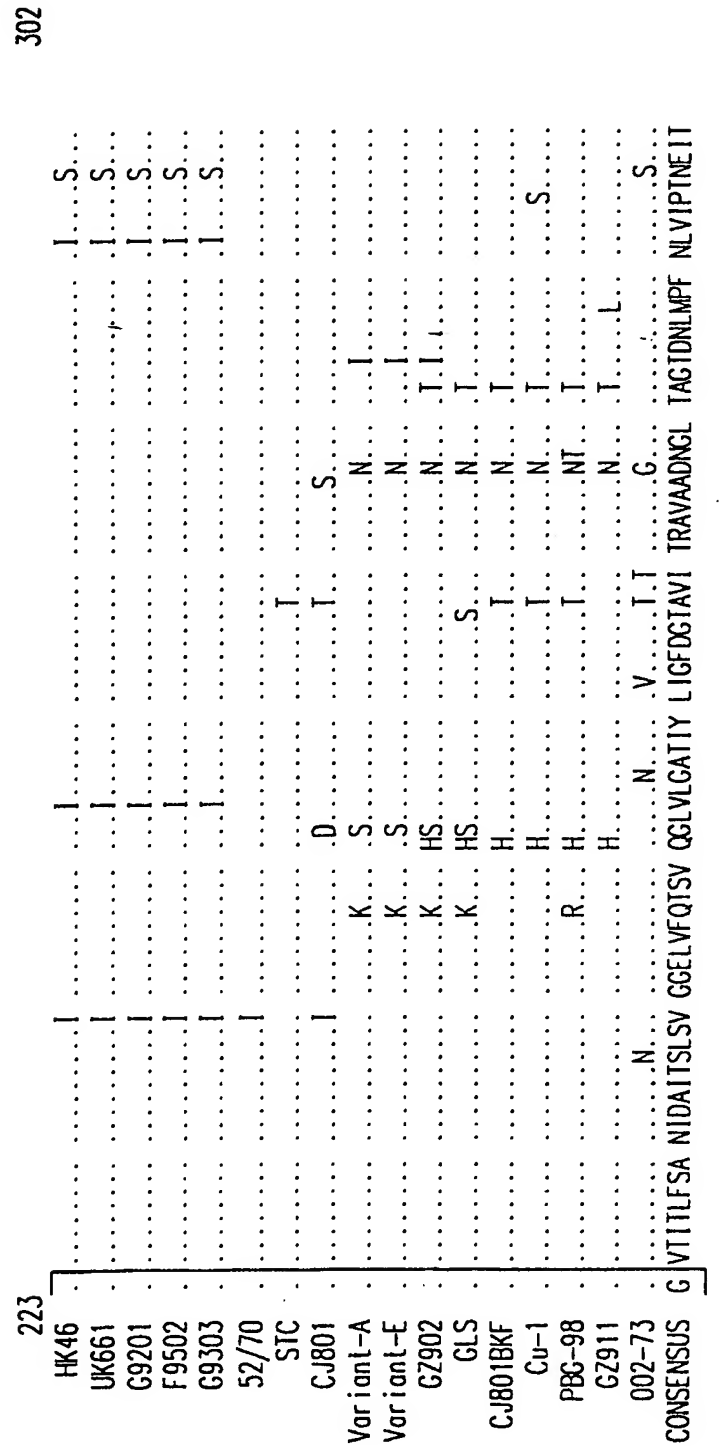
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FIG.1B

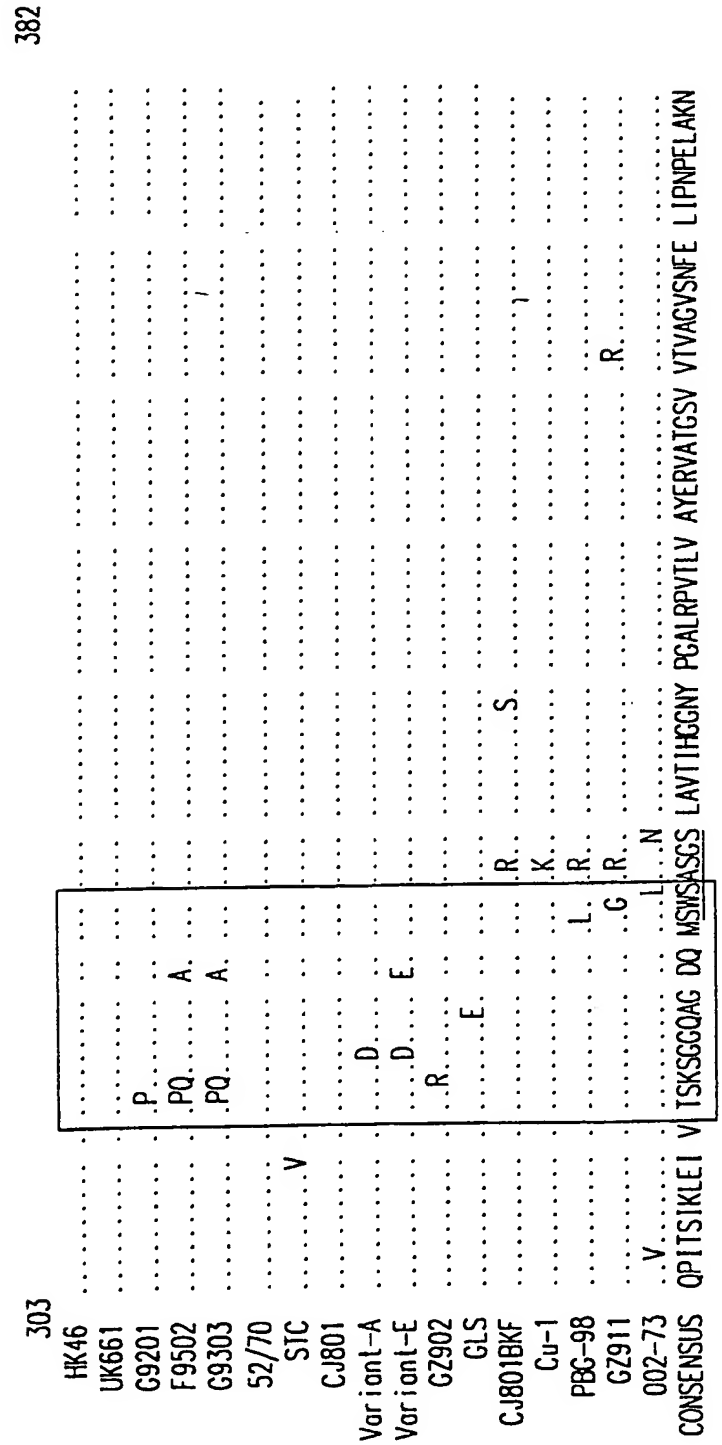
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FIG.1C

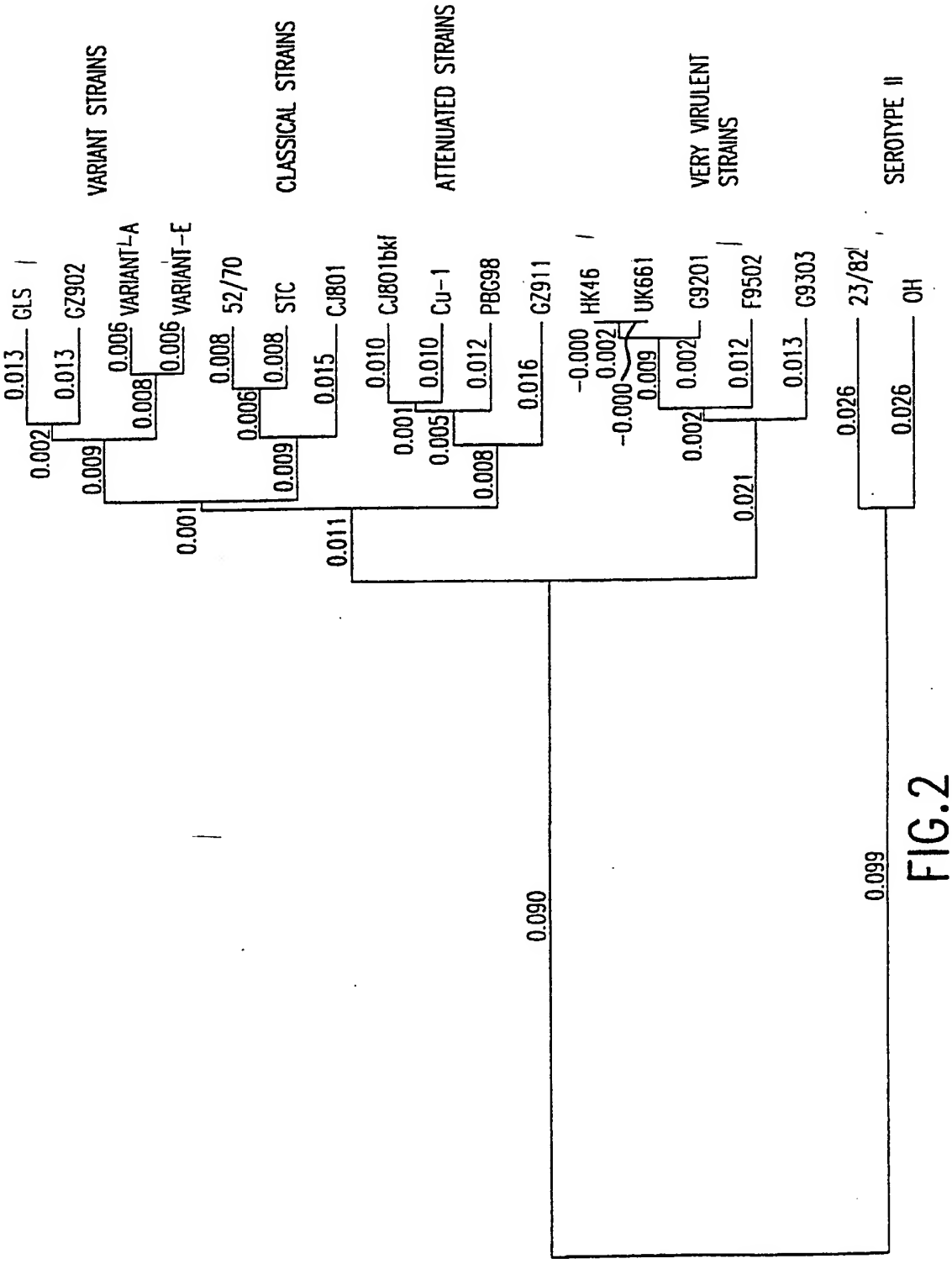
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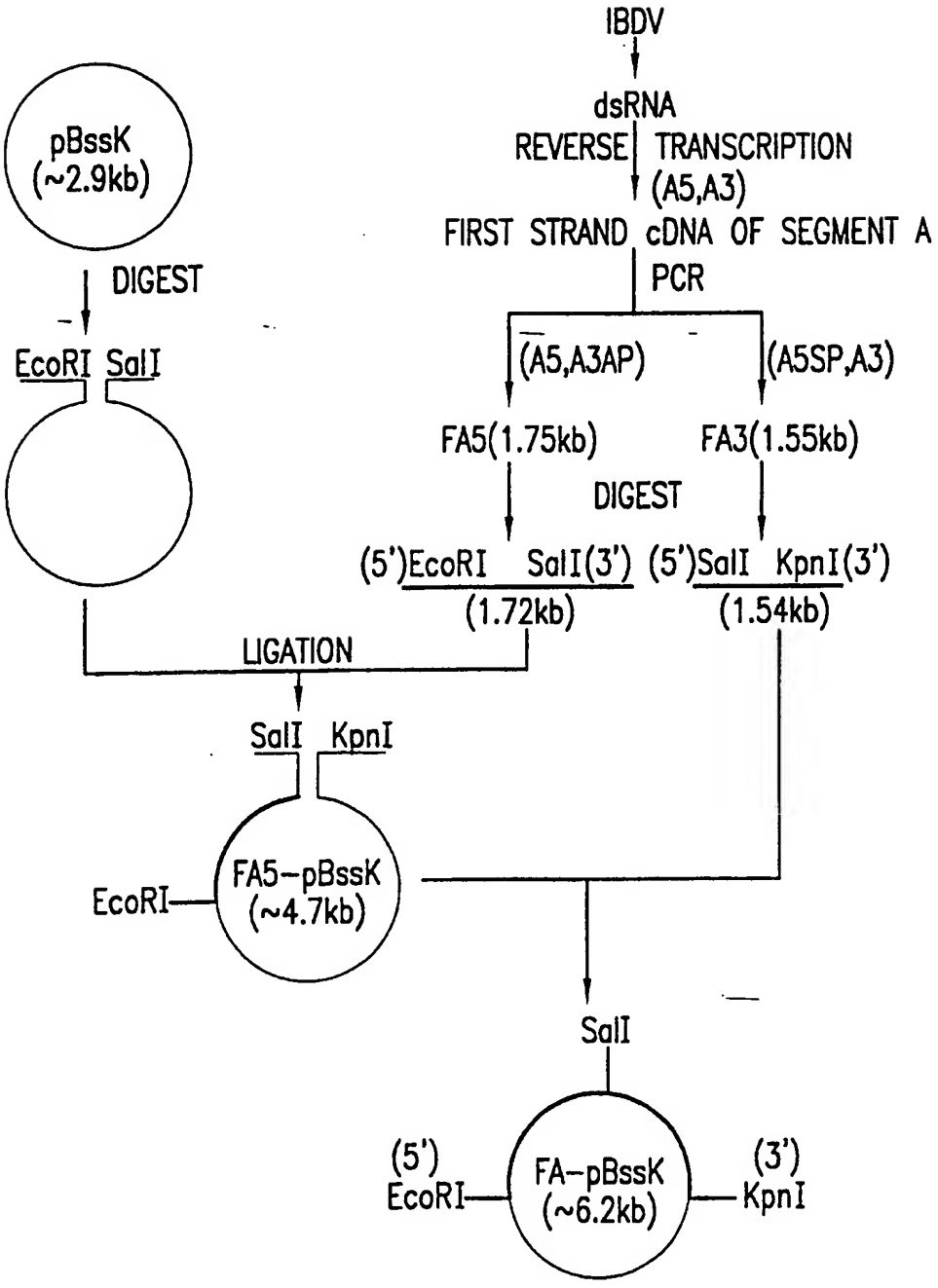
FIG.2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FA3(1.55kb)
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(1.72kb)
(5')SalI KpnI(3')
(1.54kb)
pBssK
(~2.9kb)
DIGEST
EcoRI SalI
LIGATION
SalI KpnI
EcoRI
FA5-pBssK
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(3')
KpnI

FIG.3

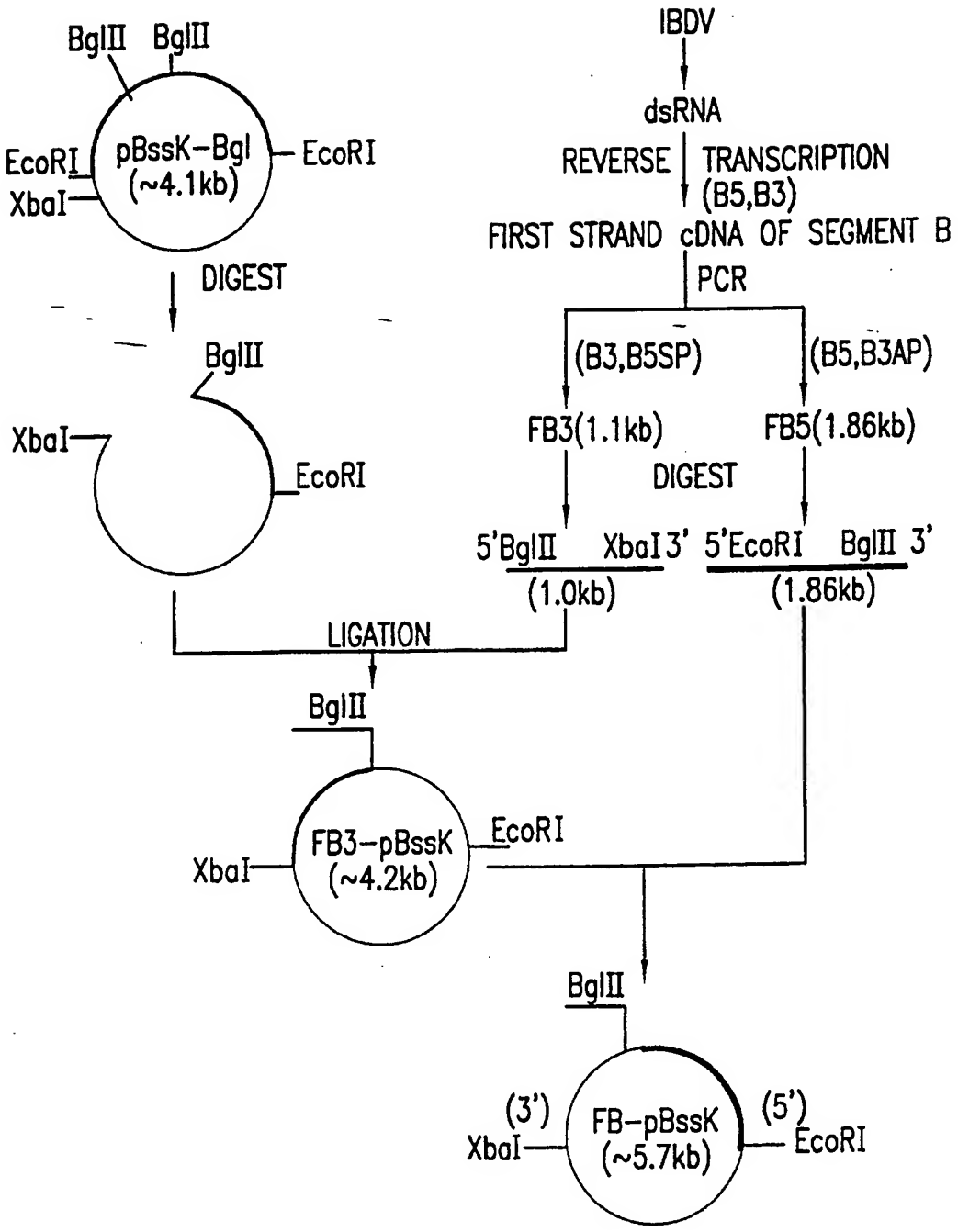
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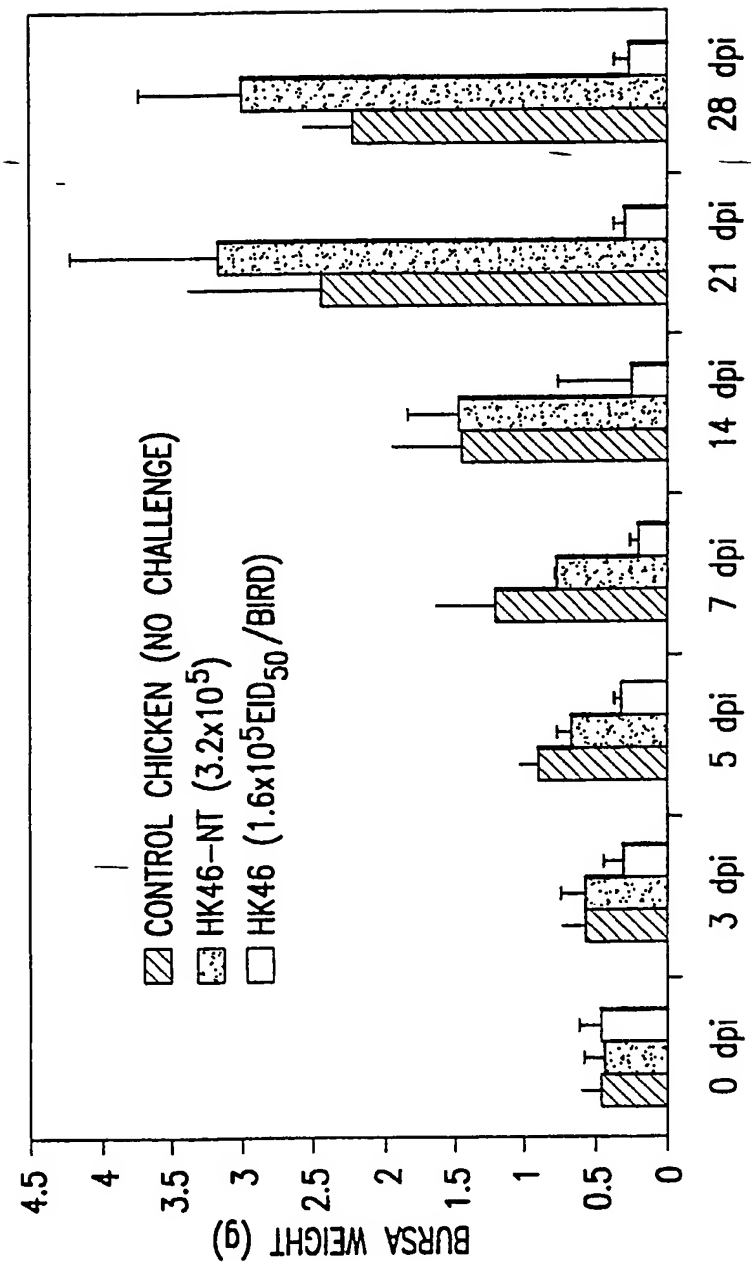
FIG.4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7 dpi
14 dpi
21 dpi
28 dpi
DAYS POST-INFECTION
CONTROL CHICKEN (NO CHALLENGE)
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HK46 (1.6x10^5EID50/BIRD)

FIG.5

SEQUENCE LISTING

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<120> GENERATION OF RECOMBINANT INFECTIOUS BURSAL DISEASE VIRUSES BY REVERSE GENETICS TECHNOLOGY AND THE USE OF THE RECOMBINANT VIRUSES AS ATTENUATED VACCINES

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<213> Artificial Sequence

<220>
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gtcactctac aagttcaac 19

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<400> 31
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<213> Infectious bursal disease virus

<400> 32

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Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr
            20                  25                  30

Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr
        35                  40                  45

Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro
    50                  55                  60

Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Ser Asn Gly Asn Tyr
65                  70                  75                  80

Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr
                85                  90                  95

Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr
            100                 105                 110

Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr
        115                 120                 125

Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu
    130                 135                 140

Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val
145                 150                 155                 160

Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly
                165                 170                 175

Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys
            180                 185                 190

Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile
        195                 200                 205

Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Ala Gly Gly
    210                 215                 220

Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu
225                 230                 235                 240

Ser Ile Gly Gly Glu Leu Val Phe Gln Thr Ser Val Gln Gly Leu Ile
                245                 250                 255

Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Ala Val Ile
            260                 265                 270

Thr Arg Ala Val Ala Ala Asp Asn Gly Leu Thr Ala Gly Thr Asp Asn
        275                 280                 285

Leu Met Pro Phe Asn Ile Val Ile Pro Thr Ser Glu Ile Thr Gln Pro
    290                 295                 300
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Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln
305                 310                 315                 320

Ala Gly Asp Gln Met Ser Trp Ser Ala Ser Gly Ser Leu Ala Val Thr
                325                 330                 335

Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val Thr Leu Val
            340                 345                 350

Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val Ala Gly Val
        355                 360                 365

Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val
    370                 375                 380

Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu
385                 390                 395                 400

Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr
                405                 410                 415

Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu
            420                 425                 430

Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile
        435                 440                 445

Arg Ala Leu Arg Arg Ile Ala Val Pro Val Val Ser Thr Leu Phe Pro
    450                 455                 460

Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val Asp Tyr Leu
465                 470                 475                 480

Leu Gly Asp Glu Thr Gln Ala Ala Ser Gly Thr Ala Arg Ala Ala Ser
                485                 490                 495

Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu Thr Leu Ala
            500                 505                 510

Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln Val Pro Gln
        515                 520                 525

Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Ile Leu Arg Gly
    530                 535                 540

Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr Leu Phe Pro
545                 550                 555                 560

Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys Ala Leu Asn
                565                 570                 575

Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp Leu Gln Pro
            580                 585                 590

Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly His Arg Val
        595                 600                 605
```

```
Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr Gly Arg Asp
    610                 615                 620

Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser Ile Met Leu
625                 630                 635                 640

Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly Thr Leu Ala
                645                 650                 655

Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile His Val Ala
            660                 665                 670

Met Thr Gly Ala Leu Asn Ala Tyr Gly Glu Ile Glu Asn Val Ser Phe
        675                 680                 685

Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu Lys Leu Ala
    690                 695                 700

Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Ser Asn Trp Ala Thr Phe
705                 710                 715                 720

Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg Leu Pro Tyr
                725                 730                 735

Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln Tyr Asp Leu
            740                 745                 750

Ala Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu Glu Ser Ala
        755                 760                 765

Val Arg Ala Met Glu Ala Ala Ala Asn Val Asp Pro Leu Phe Gln Ser
    770                 775                 780

Ala Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile Val Thr Asp
785                 790                 795                 800

Met Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg Met Arg Asn
                805                 810                 815

Phe Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln Arg Ala Lys
            820                 825                 830

Tyr Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro Thr Pro Glu
        835                 840                 845

Glu Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys Met Glu Thr
    850                 855                 860

Met Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu Asn Gly His
865                 870                 875                 880

Arg Gly Pro Ser Pro Gly Gln Leu Lys Tyr Trp Gln Asn Thr Arg Glu
                885                 890                 895

Ile Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His Ala Glu Lys
            900                 905                 910
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Ser Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala Thr Ser Ile
        915                 920                 925

Tyr Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe Ile Asp Glu
    930                 935                 940

Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro Asn Gln Glu
945                 950                 955                 960

Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys His Arg Asn
                965                 970                 975

Pro Arg Arg Ala Pro Pro Lys Pro Lys Pro Lys Pro Asn Val Pro Thr
            980                 985                 990

Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Ala Val Ser Asp
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Glu Asp Leu Glu
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<210> 33
<211> 879
<212> PRT
<213> Infectious bursal disease virus

<400> 33

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Ala Phe Gly Ile Lys Pro Thr Val Gly Gln Asp Val Glu Glu Leu Leu
             20                  25                  30

Ile Pro Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser
         35                  40                  45

Arg Leu Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Ile Leu Gln Pro
     50                  55                  60

Arg Ser Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro
 65                  70                  75                  80

Asp Leu Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr
                 85                  90                  95

Leu Ser Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro
            100                 105                 110

Thr His Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile
        115                 120                 125

Ala Leu Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala
    130                 135                 140

Thr Asp Asn Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg
145                 150                 155                 160
```

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Asp Lys Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Thr Arg Leu
                165                 170                 175

Val Ala Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro
            180                 185                 190

Leu Lys Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile
        195                 200                 205

Thr Leu Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro
    210                 215                 220

Leu Thr Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp
225                 230                 235                 240

Gly Glu Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser
                245                 250                 255

Ser Ser Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly
            260                 265                 270

Glu Met Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Ala Leu
        275                 280                 285

Leu Lys Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu
    290                 295                 300

Ser Met Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro
305                 310                 315                 320

Lys Ala Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn
                325                 330                 335

Ile Trp Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr
            340                 345                 350

Trp Pro Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly
        355                 360                 365

Cys Pro Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg
    370                 375                 380

Ile Val Glu Trp Ile Met Ala Pro Asp Glu Pro Lys Ala Leu Val Tyr
385                 390                 395                 400

Ala Asp Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp
                405                 410                 415

Leu Glu Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala
            420                 425                 430

Met Tyr Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met
        435                 440                 445

Phe Asn Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu
    450                 455                 460
```

```
Val Val Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr
465                 470                 475                 480

Gly Gln Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu
                485                 490                 495

Gly Thr Leu Val Leu Asp Gln Trp Asn Leu Met Lys Gln Pro Ser Pro
            500                 505                 510

Asp Ser Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe
        515                 520                 525

Lys Ile Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu
    530                 535                 540

Val Pro Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu
545                 550                 555                 560

Gln Pro Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr
                565                 570                 575

Tyr Ser Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg
            580                 585                 590

Leu Phe Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu
        595                 600                 605

Lys Ser Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu
    610                 615                 620

Ala Leu Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala
625                 630                 635                 640

Cys Lys Asn Asn Ala Ser Ala Ala Arg Arg His Leu Glu Ala Lys Gly
                645                 650                 655

Phe Pro Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe
            660                 665                 670

Gly Glu Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Pro Glu
        675                 680                 685

Ser Leu Ala Glu Leu Asn Arg Pro Val Pro Pro Lys Pro Pro Asn Val
    690                 695                 700

Asn Arg Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu
705                 710                 715                 720

Lys Thr Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu
                725                 730                 735

Leu Ala Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala
            740                 745                 750

Glu Ala Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp
        755                 760                 765
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Trp Phe Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp
    770                 775                 780

Ile Ala Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala
785                 790                 795                 800

Leu Glu Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu
                805                 810                 815

Val Lys Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu
            820                 825                 830

Pro Ala Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly
        835                 840                 845

Thr Ser Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala
    850                 855                 860

Val Lys Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg Gln
865                 870                 875
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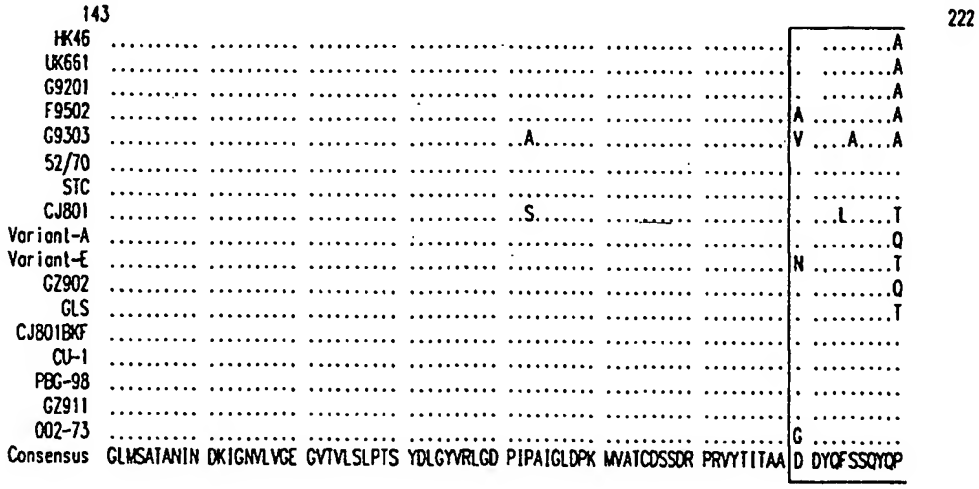

(57) Abstract

The invention relates to the generation and mutagenesis of recombinant infectious bursal disease virus by reverse genetics technology. Site-directed mutagenesis of certain amino acid residues on the Non-CEF adapted, very virulent strains of IBDV transforms the viruses into attenuated, CEF-adapted strains. The attenuated, CEF-adapted strains can be used as live vaccines against the very virulent strains. Curently available live attenuated vaccines were generated by serial passage through cell culture and the like, whereas the present invention provides a novel molecular mechanism in manipulation of the viral genome and the generation of attenuated vaccines by site-directed mutagenesis. The same approach can be used to produce vaccine strains from newly evolved IBDV viruses or to manipulate the antigenicity and pathogenicity of the virus.

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BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB99/01604

A. CLASSIFICATION OF SUBJECT MATTER

IPC[7] C12N15/00, C12N15/10, C07K13/00, A61K39/15

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched(classification system followed by classification symbols)

IPC[7] C12N15/00, C12N15/10, C07K13/00, A61K39/15

Documentation searched other than minimum documentation to the extent that such documents are included in the field searched

Chinese Patnets, Chinese Scientific and Technical Journals

Electronic data base consulted during the international search(name of data base and, where practicable, search terms used)

GenBank, EMBL, DDBJ, PDB, SwissProt, Spupdate, PIR,WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant claim No.
X	WO 91/16925 (UNIVERSITY OF MARYLAND AT COLLEGE PARK) 14 November 1991, see whole document	1-24
Y	WO 86/07060 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION) 4 December 1986, see whole document	1-24
Y	WO 98/09646 (UNIVERSITY OF MARYLAND - BIOTECHNOLOGY INSTITUTE) 12 March 1998, see whole document	1-24
A	WO 95/26196 (UNIVERSITY OF MARYLAND COLLEGE PARK) 5 October 1995, see whole document	1-24

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason(as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2000 (28. 02. 2000)	16 MARCH 2000 (16.03.00)

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB99/01604

Patent document cited in search report	Publication date	Patent family members	Publication date
WO 91/16925	14-11-91	US 5595912	21-01-97
		EP 0481072	22-04-92
WO 86/07060	04-12-86	EP 0222842	27-05-87
		AU 5966886	24-12-86
		JP 9316094	09-12-97
WO 98/09646	12-03-98	AU 3891897	26-03-98
WO 95/26196	05-10-95	AU 2129195	17-10-95
		JP 9510873	04-11-97
		US 5788970	04-08-98